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The mechanism of starch content increase in grain of autotetraploid rice (*Oryza sativa* L.)

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Abstract

Net photosynthetic rate (P_N), photorespiration (P_R), chlorophyll (Chl) content, Chl fluorescence parameters, starch accumulation, and related key enzyme activities were determined during the grain-filling stage in two autotetraploid lines and corresponding diploid rice lines. The results showed that autotetraploid rice lines had a higher Chl content, P_N, electron transport rate, maximum photochemical efficiency of PSII, actual photochemical efficiency of PSII, and lower P_R in leaves than that in corresponding diploid rice lines during the grain-filling stage. It indicated that autotetraploid rice line had a high photosynthetic capacity and high light-utilization efficiency. The activities of ADP-glucose pyrophosphorylase, soluble starch synthase, and starch-branching enzyme in grains of autotetraploid rice lines were higher than those in grains of corresponding diploid rice lines during the grain-filling stage. Therefore, autotetraploid rice lines were more efficient than corresponding diploid rice lines in converting photosynthetic products into starch.

Additional key words: 1000-grain mass; breeding; colchicine; photoassimilate; polyploidy.

Introduction

Polyploidy is widely accepted to play an important role in the evolution and breeding of plant species (Wendel 2000, Rieseberg and Willis 2007). More than 70% of angiosperms have undergone at least one round of genome duplication during the course of their evolution (Otto and Whitton 2000, Vandepoele *et al*. 2003, Hegarty and Hiscock 2008). Polyploid plants exist extensively in nature with the advantages of a larger plant size, better quality, strong adaptive ability, and high biomass yield (Birchler *et al*. 2003, Tu *et al*. 2007, He *et al*. 2011, Yang *et al*. 2014). Some important crops, such as wheat, cotton, and rape, are polyploid, and their yields doubled when their genomes were duplicated (Rieseberg and Willis 2007).

Rice (*Oryza sativa* L.) is one of the most important crops for feeding more than half population all over the world. The demand for increasing rice production is particularly urgent, because the population of traditional rice-producing countries requires more rice (Swaminathan 2007). Therefore, it is important for increasing rice production to meet the growing demand. In rice grains, starch is the predominant storage substance that accounts for about 80% of the total dry mass. It has been well documented that rice yield is mainly determined by the starch content (Asaoka *et al*. 1985, Reddy *et al*. 1994, Han and Hamaker 2001, Awika 2011). Recent work showed that multiple factors contribute to the formation of starch, including photosynthesis, and enzymes relevant to starch synthesis (Ball *et al*. 1998, Nakamura *et al*. 1989, Nakamura and Yuki 1992, Wang *et al*. 2015). ADP-glucose pyrophosphorylase (ADPG-Ppase), soluble starch synthase (SSS), and starch-branching enzyme (SBE) are the key enzymes involved in starch synthesis in the rice grain (James *et al*. 2003, Jeon *et al*. 2010). Although extensive studies have been carried out on the photosynthesis and enzymes related to starch synthesis in diploid rice, little is known about the variation in photosynthesis and enzyme activities relevant to starch synthesis when their genomes were duplicated. In the present study, two diploid rice lines and corresponding autotetraploid lines were used to explore the increase mechanism for the starch content in grains of autotetraploid rice lines.

Materials and methods

Plant materials: Two autotetraploid rice (*Oryza sativa* L.) lines SP-4X, 630-4X, and corresponding diploid rice lines SP-2X, 630-2X (Yang *et al*. 2014) were used in our experiment. Two autotetraploid rice lines (SP-4X and 630-4X) were induced by colchicine-doubling from the diploid rice lines (SP-2X and 630-2X) as described by Tu *et al*. (2007) with some modifications. Young panicles of diploid rice lines at 0.5 to 2-cm long were cut into pieces and

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Abbreviations: ADPG-Ppase – ADP-glucose pyrophosphorylase; Chl – chlorophyll; ETR – electron transport rate; F_v/F_m – maximum photochemical efficiency of PSII; P_N – net photosynthetic rate; P_R – photorespiration; SBE – starch-branching enzyme; SSS – soluble starch synthase; Φ_{PSII} – actual photochemical efficiency of PSII.
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cultured in the medium containing Murashige and Skoog (MS) nutrients plus 1 mg(2,4-dichlorophenoxyacetic acid; 2,4-D) L^{-1}, 0.2 mg(kinetin) L^{-1}, and 0.2 mg(indole-3-acetic acid) L^{-1} for callus induction. The induced callus was implanted on a secondary medium containing MS plus 0.4mg(vitamin B_1) L^{-1}, 2mg(2,4-D) L^{-1}, 100mg(inositol) L^{-1}, and 25 g(mannitol) L^{-1}, three times for 20 d. Uniformly light yellow callus tissues with vigorous growth were selected, cut into 2-mm pieces, and grown in the secondary medium plus 500 mg(colchicine) L^{-1} for 48 h at 25°C in shaking culture. The callus tissues were filtered with a sterile 0.5-μm screen, washed with culture fluid to remove residual colchicine, and cultured in the regenerating medium containing MS plus 2 mg(6-benzylaminopurine) L^{-1}, 0.5 mg(kinetin) L^{-1}, and 1 mg(1-naphthaleneacetic acid) L^{-1} for inducing shoots. Finally, the callus tissue with shoots was transferred to rhizogenic medium containing 0.5 MS plus 1 mg(vitamin B_1) L^{-1} and 0.5 mg(3-indolebutyric acid) L^{-1} for 20 d. Plants with vigorous roots were transplanted to soil and subsequently into the field. Autotetraploids were selected based on cytological verification.

Experimental design: Field experiment was conducted at the experimental farm in Xinxiang Academy Agricultural Science, Xinxiang, China (35°18'N, 113°52'E). The diploid rice lines and autotetraploid rice lines were grown in 2017 (from May to October) under the same field conditions. The seeds used for tests were first sown in a seedling bed to promote germination and cultivate young seedlings for 30 d, and then the young seedlings were transplanted to a paddy field. Each rice line comprised of 20 rows, and each row was for 40 individuals; the spacing between the rows was 30 cm, and the spacing between individuals in a row was 15 cm. Two hundred grains were sampled at 15, 30, 45 d, respectively, after anthesis from the middle of ears and divided into two groups. One group (100 grains) was frozen in liquid nitrogen and kept at –80°C for enzyme assays. Another group (100 grains), after their enzymes were deactivated at 105°C for 30 min, was dried at 80°C to constant mass, and then milled, passed through a 100-mesh sieve and kept in desiccator for starch assays.

Chl content: Leaf Chl content was measured following the method of Arnon (1949) and expressed as mg g^{-1}(fresh mass, FM). Samples of approximately 0.5 g were cut from mid-section of fresh flag leaves. Each sample was grinded in 5 mL of 80% acetone (with little $CaCO_3$ and quartz sand), and then stored in dark for 5 min. The solution was filtered into 50-mL volumetric flask. The residues were grinded and filtered again in the same manner. Finally, the volumetric flasks were made up to the mark with 50 mL by 80% acetone. The absorbance of the extract was estimated at 645 and 663 nm using 80% acetone as a blank with a spectrophotometer *UV-4802* (*Yuanxi Co.,* Shanghai, China). The Chl content was calculated using the following equations: total Chl = [20.29 (A_{645}) + 8.04 (A_{663})] × (V/1000 M), where V was the volume of the extracted liquid, and M was the fresh mass of the sample.

P_N* and *P_R: P_N was measured under the conditions of natural environment (field) on sunny day. We also recorded changes in PFD and temperature in Xiangxiang at this time (Fig. 1). *LI6400* portable photosynthesis system (*LI-COR Co*., USA) was used to measure the P_N of flag leaves at 15, 30, 45 d, respectively, after anthesis under natural conditions at 07:00, 09:00, 11:00, 13:00, and 15:00 h. P_R was calculated as described by Guan *et al*. (2004). The P_N was measured at O_2 concentration of about 1% in order to inhibit the P_R. This was achieved by mixing nitrogen gas with normal air in a mixing ratio that resulted in about 1% O_2 which was monitored with an oxygen electrode. P_R was estimated by subtracting P_N under normal O_2 concentration from that under 2% O_2. Each result shown was the mean of ten replicated treatments.

The Chl fluorescence parameters of flag leaves were measured with a portable Chl fluorometer (*Mini-PAM*, *Heinz Walz GmbH*, Effeltrich, Germany). The mean values of leaf electron transport rate (ETR), maximum photochemical efficiency of PSII (F_v/F_m), and actual photochemical efficiency of PSII (Φ_{PSII}) were measured as described by Baker (2008). The mean value of each leaf ETR, F_v/F_m, and Φ_{PSII} was determined by measuring ten leaf samples.

Preparation and assay of enzymes: Twenty dehulled rice grains were hand-homogenized at 4°C in a mortar and pestle with 5 mL of extraction buffer containing 100 mmol Tricine-NaOH, pH 7.5, 8 mmol $MgCl_2$, 2 mmol EDTA, 12.5% (v/v) glycerol; 1% (w/v) PVP-40, and 50 mmol 2-mercaptoethanol. After centrifugation at 10,000 × *g* for 25 min, the supernatant solution and deposition were

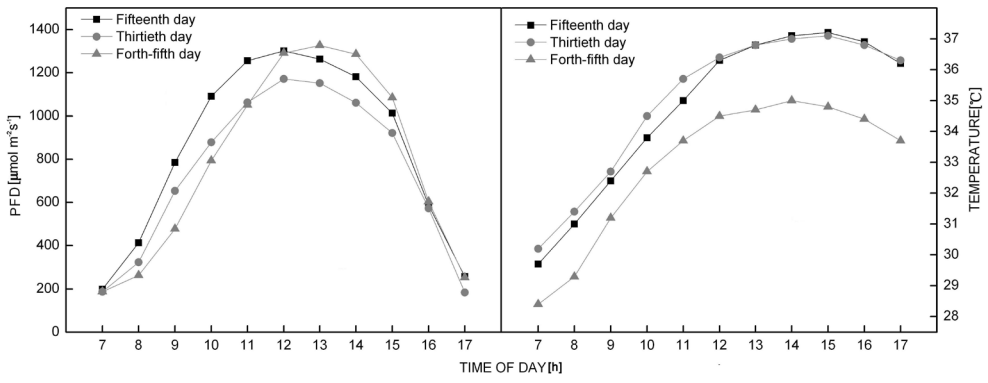

Fig. 1. Diurnal changes of temperature and light intensity during the grain-filling stage of rice.

collected separately and used as the preparation for individual enzyme analysis. ADP-glucose pyrophosphorylase (ADPG-Ppase, EC 2.7.7.27) and soluble starch synthase (SSS, EC 2.4.1.21) activities were measured as described by Xie *et al*. (2016). To determine ADP-glucose pyrophosphorylase activity, 20 μL of soluble crude enzyme was taken from samples and added into 230 μL of reaction liquid consisting of 100 mmol L^{-1} Hepes-NaOH (pH 7.4), 3 mmol L^{-1} 3-PGA, 1.2 mmol L^{-1} ADPG, 3 mmol L^{-1} NaH_2PO_4, 5 mmol L^{-1} $MgCl_2$, 4 mmol L^{-1} DTT, 15 μL of distilled water. The homogenate was vibrated for 20 min at 30°C, and the reaction terminated by immersion for 30 s in 100°C boiling water. The resulting supernatant was moved into a 3-mL quartz cuvette to which 450 μL of distilled water was added. It was centrifuged at 15,000 rpm min^{-1} in the small tube for 10 min at 2°C. Then, 15 μL of of 10 mg mL^{-1} NADP was added. This was vibrated with film. Absorbance at a wavelength of 340 nm was measured using an ultraviolet spectrophotometer. This spectral absorbance was re-measured after adding 1 μL of PGM and 1 μL of G-6-PDH.

To determine soluble starch synthase activity, 40 μL of soluble crude enzyme was taken from samples and added to a 140 μL of reaction liquid consisting of 50 mmol L^{-1} Hepes-NaOH (pH 7.4), 15 mmol L^{-1} DTT, 1.6 mmol L^{-1} ADPG, 20 mg mL^{-1} glycogen, and 100 μL of distilled water. The homogenate was vibrated for 40 min at 30°C and the reaction terminated by immersion for 2 min in 100°C boiling water. Then, 100 μL of another reaction liquid (50 mmol L^{-1} Hepes-NaOH (pH 7.4), 4 mmol L^{-1} PEP, 200 mmol L^{-1} KCl, 10 mmol L^{-1} $MgCl_2$, 2 μL of pyruvate kinase) was added to this solution and continued vibration for 30 min at 30°C. This reaction was terminated by immersion for 2 min in 100°C boiling water. Then 100 μL of distilled water was added to this solution, with the resulting supernatant placed in a 3-mL quartz cuvette that was then centrifuged at 15,000 r min^{-1} for 10 min at 2°C, and added into 200 μL of a solution containing 50 mmol L^{-1} Hepes-NaOH (pH 7.4), 20 mmol L^{-1} $MgCl_2$, 10 mmol L^{-1} glucose, 2 mmol L^{-1} NADP, and 20 μL of distilled water. This mixture was then vibrated with film. Absorbance at a wavelength of 340 nm was measured using an ultraviolet spectrophotometer. This spectral absorbance was re-measured after adding 2 μL of G-6-PDH.

Starch-branching enzyme (SBE, EC 2.4.1.18) activity was measured as described by Nakamura *et al*. (1989). ADPG-Ppase, SSE, and SSS activities were expressed as μmol s^{-1} mg^{-1}(protein). Each result shown was the mean of three replicated treatments.

The starch content in grain of rice was measured using the methods of Fujita *et al*. (2003).

Statistical analysis: All the data in the present study were expressed as means ± SE. Significance analysis was performed using *SAS* software (*SAS Institute*, *Inc.*, Cary, NC, USA). One-way analysis of variance (*ANOVA*) method (*Duncan*'s multiple range test) was used to detect the significance (P<0.05).

Results

Chl content: During the grain-filling stage, the Chl content of autotetraploid rice line was higher than that of corresponding diploid rice line, but the Chl content showed no significant differences between two autotetraploid rice lines and corresponding diploid rice lines in the early stage of grain filling (Fig. 2). The Chl content in flag leaves of all rice lines decreased with the prolongation of grain filling time (Fig. 2), but the Chl content in flag leaves of diploid rice line decreased sharper then those of corresponding autotetraploid rice line. The Chl content showed significant difference between autotetraploid rice and corresponding diploid rice in the late stage of grain filling (Fig. 2).

P_N* and *P_R: During the grain-filling stage, the P_N of autotetraploid rice line was higher than that of corresponding diploid rice line, but the difference of P_N between autotetraploid rice line and corresponding diploid rice line was not significant in the early stage of grain filling (Fig. 3). The P_N difference between autotetraploid rice line and corresponding diploid rice line increased with prolongation of grain filling time. The P_N of autotetraploid rice line was significantly higher than that of corresponding diploid rice line in the late stage of grain filling. During grain-filling stage, the P_R of autotetraploid rice line was lower than that of corresponding diploid rice line and the difference of photorespiration between autotetraploid rice line and corresponding diploid rice line was significant in the early stage of grain filling (Fig. 3).

Chl fluorescence parameters: The F_v/F_m, Φ_{PSII}, and ETR

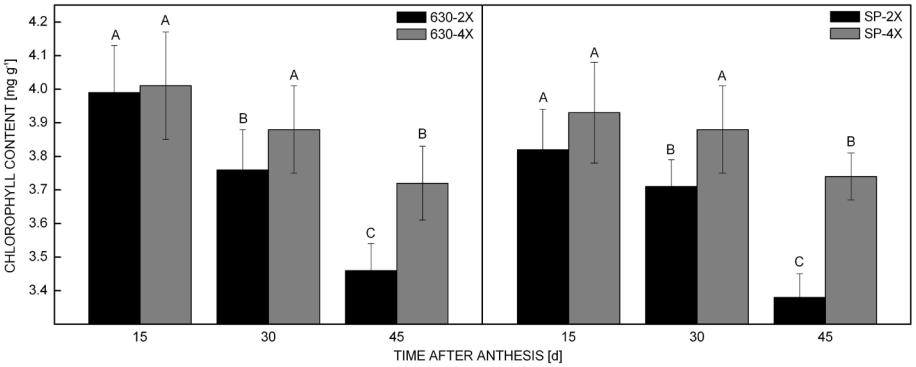

Fig. 2. Chlorophyll content in flag leaves of diploid and autotetraploid rice lines during the grain-filling stage. Error bars show SE, n = 3. One-way analysis of variance (*ANOVA*) was used to indentify differences between diploid and corresponding autotetraploid in each time. Values with *different letters* are significantly different (P<0.05). 2X – diploid rice line, 4X – auto-tetraploid rice line.

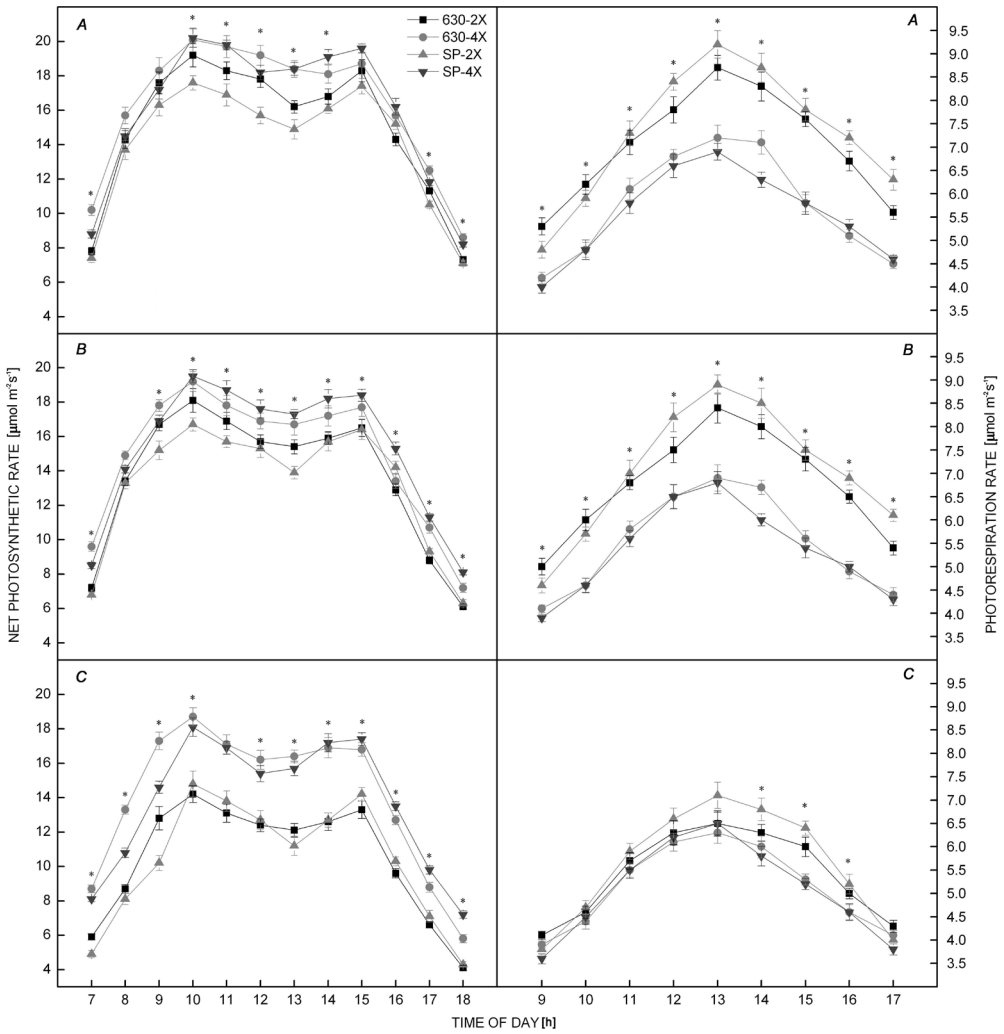

Fig. 3. Changes of net photosynthetic rate (P_N) and photorespiration (P_R) in diploid and autotetraploid rice lines during the grain-filling stage: 15 d after anthesis (*A*); 30 d after anthesis (*B*); and 45 d after anthesis (*C*). Error bars show SE, $n = 10$. *Asterisks* indicate significant difference ($P<0.05$) between two diploid rice lines and corresponding autotetraploid rice lines. 2X – diploid rice line, 4X – autotetraploid rice line.

of autotetraploid rice line were higher than those of corresponding diploid rice line during grain-filling stage, and the F_v/F_m, Φ_{PSII}, and ETR showed significant differences between autotetraploid rice line and corresponding diploid rice line during late stage of grain-filling process (Fig. 4). The F_v/F_m, Φ_{PSII}, and ETR of all rice line decreased with the prolongation of grain filling time, but F_v/F_m, Φ_{PSII} and ETR of diploid rice line decreased sharper then those of corresponding autotetraploid rice line (Fig. 4).

The activities of ADPG-Ppase, SSS, and SBE were significantly higher in autotetraploid rice line than those in corresponding diploid rice line during the grain-filling stage (Fig. 5). The activities of ADPG-Ppase, SSS, and SBE decreased with the prolongation of grain filling time for all rice lines. However, ADPG-Ppase, SSS, and SBE activities in diploid rice line decreased sharper then those in corresponding autotetraploid rice line.

Starch accumulation in filling grains of diploid and autotetraploid rice line: The starch accumulations of different ploidy rice lines are showed in Fig. 6. Starch content increased with prolongation of grain filling time for all rice lines. However, the starch content in autotetraploid rice line increased sharper then those in corresponding diploid rice line, and the starch content in autotetraploid rice line was significantly higher than that in corresponding diploid rice line in maturity. Chromosome doubling had a considerable effect on the starch content in rice grains.

Discussion

Crop starch depends on the capacity of source tissues (especially flag leaves) to produce photoassimilates during the grain-filling process, as well as on the ability of sink tissues to convert this photoassimilates into starch (Reynolds *et al*. 2012, Tuncel and Okita 2013). Theoretically, crop starch can be increased by promoting photosynthesis

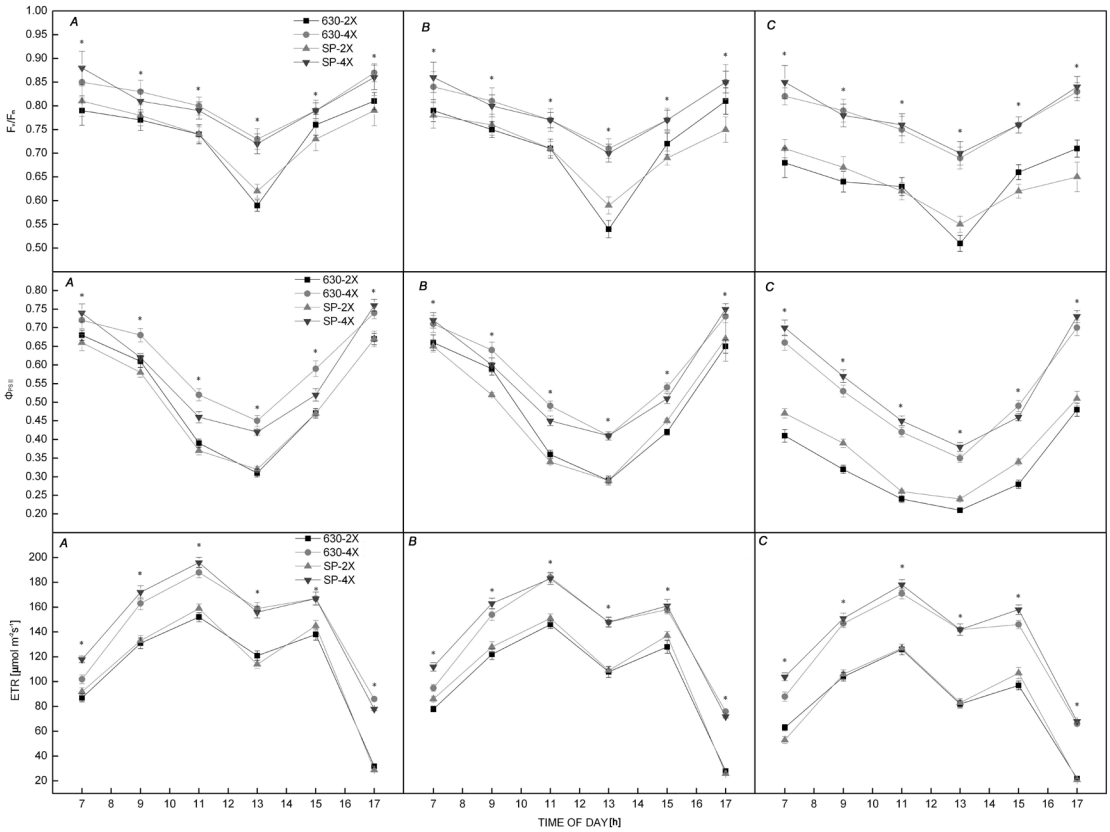

Fig. 4. Changes of chlorophyll fluorescence parameters in diploid and autotetraploid rice lines during the grain-filling stage: 15 d after anthesis (*A*); 30 d after anthesis (*B*); and 45 d after anthesis (*C*). Error bars show SE, $n = 10$. *Asterisks* indicate significant difference ($P<0.05$) between two diploid rice lines and corresponding autotetraploid rice lines. 2X – diploid rice line, 4X – autotetraploid rice line, F_v/F_m – maximum photochemical efficiency of PSII, Φ_{PSII} – actual photochemical efficiency of photosystem II, ETR – electron transport rate.

because photosynthesis is the major metabolic pathway that converts carbon dioxide into organic compounds, such as fructose, glucose, sucrose, and starch in plants (Chang *et al*. 2017). Therefore, the plant's photosynthetic efficiency and conversion capacity of photoassimilates after anthesis have become important factors for crop starch accumulation (Miralles and Slafer 2007). Polyploidization can improve photosynthetic characteristics, enzymatic activity, secondary metabolites, *etc.*, in many plants (Meng *et al*. 2014, Ghimire *et al*. 2016, Liao *et al*. 2016, Wang *et al*. 2016). In autotetraploid plant, the basic genetic material remains the same, but gene dosage is multiplied. Hence autotetraploid plant has four sets of chromosomes, carries up four alleles, many genes duplicated by polyploidy retain their original function, and there is potential to enhance trait expression (Wendel 2000), *e.g.*, improved photosynthetic characteristics have been reported in tetraploid black locust, triploid rice, *etc*. (Meng *et al*. 2014, Ghimire *et al*. 2016, Wang *et al*. 2016), the enzyme activity of potato granule-bound starch synthase is linearly correlated with dosage of allele (Flipse *et al*. 1996), the production of secondary metabolites displayed increased abundances in some polyploidy plants (Dhawan and Lavania 1996, Lavania 2005), significant upregulation of several key enzymes related to artemisinin biosynthetic pathway was observed in autotetraploid plants of *Artemisia annua* (Lin *et al*. 2011), and Ghotbi Ravandi *et al*. (2013) reported the tetraploid plants of *Cichorium intybus* with a significant increase in total phenolic compounds and chlorogenic acid content in leaves. Furthermore, genes associated with photosynthesis and enzyme activities were found to be upregulated in the autotetraploid *vs.* diploid comparison (Fan *et al*. 2015, Madani *et al*. 2015). These reports indicated the most duplicate genes retained original functions, and enhanced trait expression in polyploid plants. In our experiments, Chl content, P_N, ETR, F_v/F_m, and Φ_{PSII} were found to be upregulated in autotetraploid rice lines compared to diploid lines during the grain-filling stage, suggesting that photosynthetic capacity and photosynthetic efficiency were accelerated by increasing of the ploidy level, which would ensure sufficient photoassimilates for autotetraploid rice lines to synthesize starch. These advantages were especially obvious at the late stage of grain-filling process. Photorespiration is an inherent feature of plants. It is estimated that about 1/3 of carbon dioxide assimilation in photosynthesis is released by photorespiration (Bauwe *et al*. 2010). Photorespiration consumes photosynthesis products, resulting in a reduced production. The most interesting finding was that chromosome doubling reduced the photorespiration of

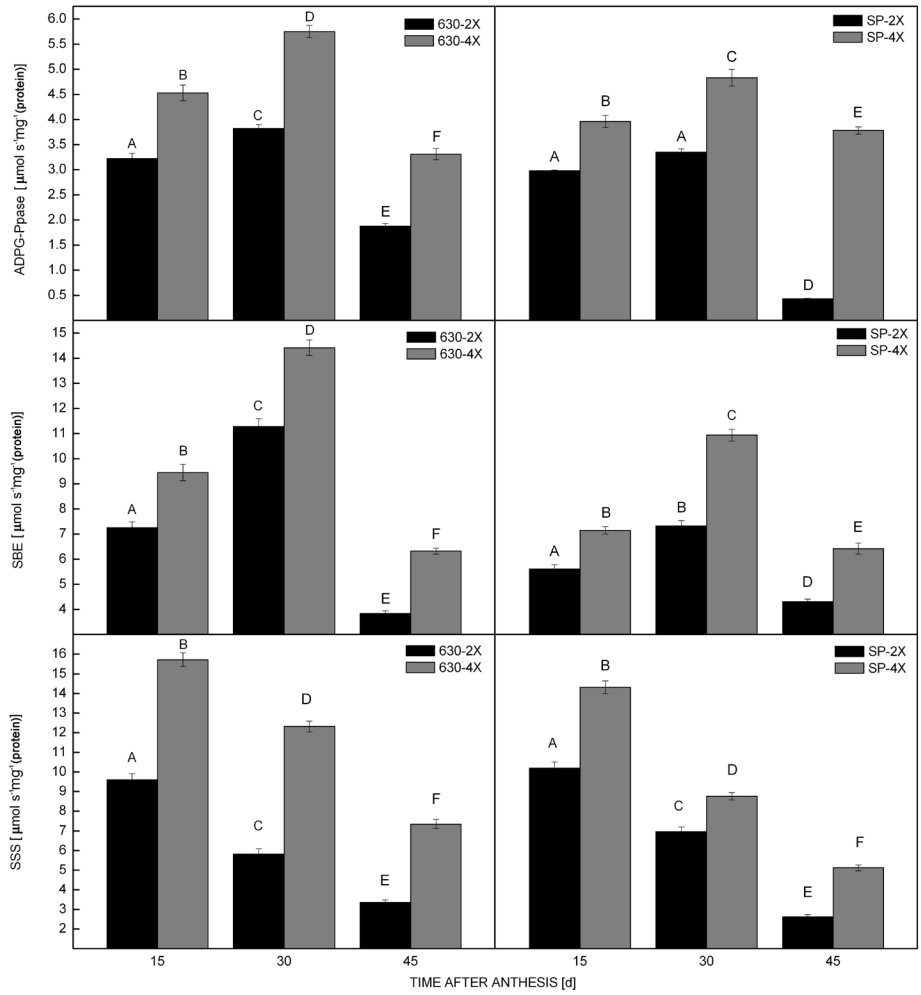

Fig. 5. The activities of ADP-glucose pyrophosphorylase (ADPG-Ppase), soluble starch synthase (SSS), and starch-branching enzyme (SBE) in grains of diploid and autotetraploid rice during the grain-filling stage. Error bars show SE, n = 3. One-way analysis of variance (*ANOVA*) was used to indentify differences between diploid and corresponding autotetraploid rice line in each treatment. Values with *different letters* are significantly different (P<0.05). 2X – diploid rice line, 4X – autotetraploid rice line.

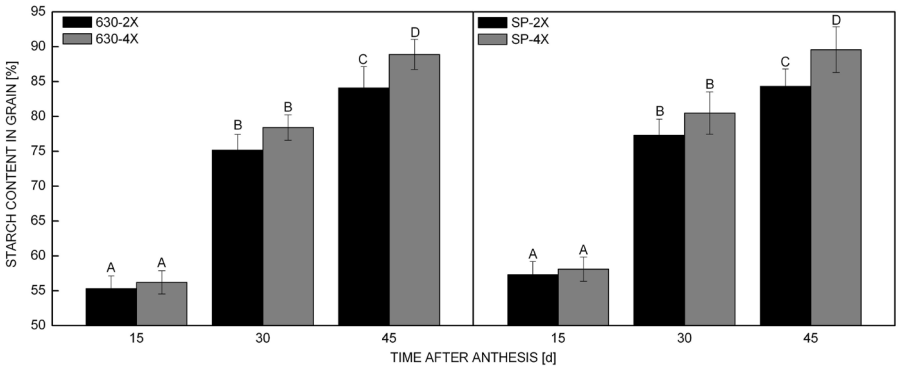

Fig. 6. Starch accumulation in grains of diploid and autotetraploid rice lines during the grain-filling stage: 15 d after anthesis (*A*); 30 d after anthesis (*B*); and 45 d after anthesis (*C*). Error bars show SE, n = 3. One-way analysis of variance (*ANOVA*) was used to indentify differences between diploid and corresponding autotetraploid rice lines in each time. Values with *different letters* are significantly different (P<0.05). 2X – diploid rice line, 4X – autotetraploid rice line.

rice. The two autotetraploid rice lines maintained lower P_R than the corresponding diploid rice lines during the grain-filling stage. During the grain-filling stage, autotetraploid rice lines maintained higher photosynthetic capacity and efficiency of light energy utilization, lower consumption of photosynthesis products than corresponding diploid lines, which could be beneficial to promote amount of photoassimilates in autotetraploid rice lines.

Starch biosynthesis in rice endosperm catalyzed by a series of enzymes can assimilate photoassimilates into starch. ADPG-Ppase, SSS, and SBE are key enzymes involved in controlling starch synthesis and accumulation (Myers *et al*. 2000, Pandey *et al*. 2012). ADPG-Ppase is the first rate-limiting enzyme in the process of starch synthesis (MacLeod and Duffus 1988, Jenner 1991, Kato 1995, Jiang *et al*. 2003). ADPG-Ppase catalyzes the synthesis of ADPG, which is the glucose donor in starch synthesis in rice. The activity of ADPG-Ppase in grains of autotetraploid rice line was greater than that in corresponding diploid rice line, indicating that more ADPG could be generated by autotetraploid rice line, which resulted in an increased supply of the ADP-glucose donor for starch synthesis

during the grain-filling stage. The glucose molecule of ADPG is transferred to the nonreducing ends of the α-1,4-glucan chain by SSS to form amylose. SSS promotes the formation of α-1,4-glycosidic bonds in the amylopectin molecules of starch particles (Larkin *et al*. 2003, Butardo *et al*. 2011). The formation of branches between α-1,6-glycosidic bonds in amylopectin is catalyzed by SBE. It promoted the enlargement of dextran molecules, meanwhile, it was favorable to the catalyzed reaction of ADPG-Ppase and SSS, so that more starch could be catalyzed and synthesized in a short time (Nakamura *et al*. 2010). The activities of ADPG-Ppase, SSS, and SBE in grains of autotetraploid rice lines were significantly higher than that in corresponding diploid rice lines during the grain-filling stage, the conversion of photoassimilates to starch was accelerated by increasing of enzyme activity related to starch accumulation in autotetraploid rice lines. It was suggested that the improved starch content in autotetraploid mainly is attributed to the increased photosynthetic capacity, photosynthetic electron transfer efficiency, and enzyme activity related to starch synthesis in the autotetraploid. After maturation, the 1000-grain masses of 630-4X and SP-4X was 50.8 and 53.1 g, respectively. Moreover, the 630-2X and SP-2X had 1000-grain masses that were 29.2 and 25.1 g, respectively (Yang *et al*. 2013). The kernel masses of two autotetraploid rice lines were significantly higher compared to those of corresponding diploid rice lines.

In conclusion, autotetraploid rice lines maintained higher Chl content, P_N, ETR, F_v/F_m, and Φ_{PSII} and lower P_R than that of the corresponding diploid lines during the grain-filling stage. These advantages increased photosynthetic capacity and utilization efficiency of light energy and reduced consumption of photosynthate products. This, in turn, accelerated photosynthate assimilation, increased amount of photosynthates to meet the needs of starch synthesis. On the other hand, the activities of ADPG-Ppase, SSS, and SBE in grains of autotetraploid rice lines were higher than that in corresponding diploid rice lines. The higher ADPG-Ppase, SSS, and SBE activity was beneficial for converting photosynthetic products into starch. Therefore, autotetraploid rice line had a higher capacity for starch synthesis than the corresponding diploid rice line. It was the reason why the starch content in grains of autotetraploid rice lines was higher than that of diploid rice lines.

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